10/051.644 09.17.03

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 49/00, C12N 15/12, 15/85, 5/10, C07K 14/435, A01K 67/033, C12Q 1/68

A2

(11) International Publication Number: WO 00/54815

(43) International Publication Date: 21 September 2000 (21.09.00)

(21) International Application Number: PCT/EP00/02373

(22) International Filing Date: 16 March 2000 (16.03.00)

(30) Priority Data:
9906018.8 16 March 1999 (16.03.99) GB

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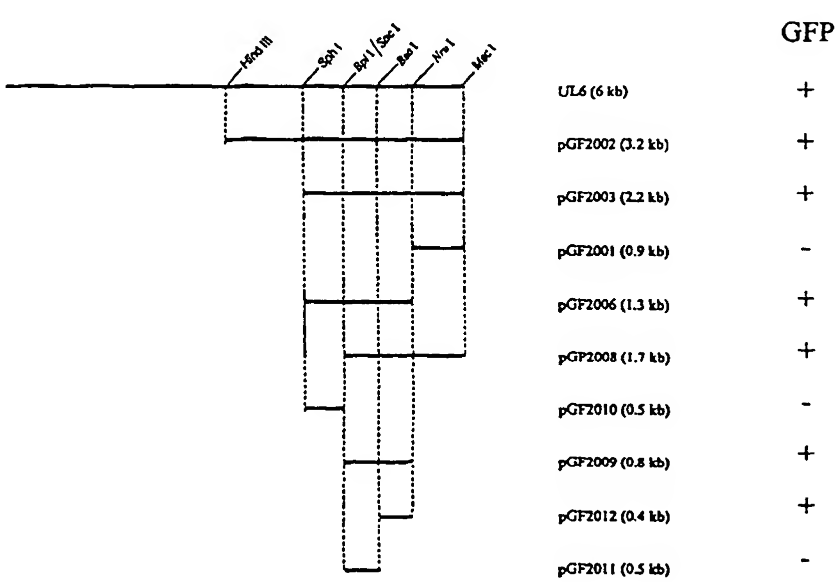
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: EXPRESSION OF DNA OR PROTEINS IN *C. ELEGANS*



(57) Abstract

DNA fragments from the promoter region of the *C. elegans* UL6 gene which are capable of functioning as promoters directing gene expression in the excretory cell of *C. elegans* are provided and also expression vectors and transgenic *C. elegans* containing these fragments. Also provided are screening methods performed in *C. elegans* for identifying compounds or mutations which have an effect on the morphology of the excretory canal. Compounds identified using these screening methods may have therapeutic potential in the treatment of a range of diseases for which the *C. elegans* excretory canal serves as a model.

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Expression of DNA or proteins in *C. elegans*.

The present invention relates to the expression of DNA, genes, cDNAs, proteins, peptides and parts thereof in the excretory canal of the nematode worm *C.elegans*. In particular, the invention relates to promoter sequences which are capable of directing tissue-specific gene expression in the excretory canal of *C. elegans*, to expression vectors containing the promoter sequences, to transgenic *C. elegans* specifically expressing reporter genes in the excretory canal, to methods of identifying chemical agents that affect the morphology of the excretory canal and to use of these agents in the pharmacological treatment of diseases for which the *C.elegans* excretory canal serves as a model.

The *C.elegans* excretory cell

The excretory system of the nematode *C.elegans* consists of three cells: a single large excretory cell, a duct cell and a pore cell that interfaces with the duct to the main body hypodermis. The excretory cell is the largest mononucleate cell in *C. elegans*. The nucleus and cell body of the excretory cell is situated at the terminal bulb of the pharynx. The cell itself is shaped in an H-form, with the two arms situated along the lateral lines for almost the entire length of the worm, and slightly dorsal. The excretory cell is polarized, having an apical domain facing the lumen of the excretory canal and a basal domain facing outside. The structure and the organization of the *C. elegans* excretory system suggest that it may be used for osmoregulation and can therefore be considered as

a model for the vertebrate nephron.

Various mutant *C. elegans* have been reported which have an aberrant phenotype in the excretory canal. These aberrant phenotypes include cyst formation, short canals and branched canals. Various mutations affecting the excretory canal can be traced back in C.elegans II, ed. Riddle, Blumenthal, Meyer and Priess, Cold Spring Harbor Laboratory Press, 1997.

Drug discovery in growth cone steering.

Regulation of cell motility, cell shape and the outgrowth of axons or other cell outgrowths are all essential processes in the morphogenesis and function of both unicellular and multicellular organisms. Furthermore, the control of these processes is disturbed in a variety of diseases in which receptors, extra-cellular signals and intra-cellular pathways are over- or under-stimulated. The discovery of new genes, proteins and peptides that are involved in these processes and chemical entities which modulate them would very much help the understanding of these processes. Accordingly, there is a need to develop new methods for the discovery of novel molecules involved in the cell motility, cell shape and cell outgrowth process, and to establish their function. In addition, since malfunction of these biological processes can lead to disease there is also a need to discover chemical entities which modulate these processes which may be useful as pharmaceuticals. Diseases associated with cell motility, cell shape and cell outgrowth include cancerous disease, more particularly tumour formation, metastasis and vascularisation of tumours.

Drug discovery in renal diseases.

In the drug discovery process it is established practice to develop a model of a disease which can be used in the development of assays to screen for compounds with potential pharmaceutical activity. For kidney diseases, and more specifically kidney cyst formation, two different types of disease models currently exist; models based on cell cultures of renal epithelial cells and mouse models. Although these systems have been presented as models for cystic diseases, such as autosomal dominant polycystic kidney disease (ADPKD), they have several disadvantages.

The models based on cell cultures can never be compared with a live multicellular organism. Where aberrant growth indicative of cyst formation has been observed in cultures of different cells, it has proven difficult to develop efficient compound screens from these models. Furthermore, even if chemicals can be discovered that modulate cell growth and hence cyst formation in culture, it remains difficult to prove that these compounds will have analogous effects in the renal systems of multicellular organisms.

The developed mouse models for renal cyst diseases have the disadvantage that they are not suitable for middle to high throughput screening for the discovery of pharmacological compounds. Accordingly, there remains a clear need for an alternative model of renal diseases which more accurately models the renal systems of multicellular organisms but which is practical for use in middle to high throughput screening.

The present invention relates to the use of the *C. elegans* excretory cell in the drug discovery

process. The *C.elegans* excretory canal is an efficient tool to study various developmental biological features; it is formed during the larval stages of the nematode and the canals are observed to grow along the animal in early development. Hence, the development of the excretory canal is an efficient tool to study growth cone steering and defects that might arise during its development and the excretory canal can be used as a model for the development of drug screens in the area of growth cone steering and directional outgrowth.

The *C. elegans* excretory cell and excretory canal can also be considered as a model of the human kidney nephron. The excretory canal has analogous apical-basal polarities as can be found in certain kidney cells and which are relevant for cellular function. Hence, studying the excretory canal may help to develop new tools against kidney diseases. Furthermore, the excretory canal can be used as a model for the development of drug screens in the area of kidney diseases.

In order to exploit the potential of the *C. elegans* excretory cell and excretory canal both as a disease model and in the development of drug screens it would be advantageous to be able to express any gene or cDNA of interest, including reporter genes, specifically in the excretory cell and excretory canal. To achieve this would require the identification of a tissue-specific promoter which is active in the excretory cell.

The present inventors have identified, through the use of biochemical, molecular biology and transgenic techniques, a promoter fragment that

specifically directs transcription in the *C. elegans* excretory cell in a very efficient way. From this promoter fragment several deletions have been generated that still promote transcription, and hence gene expression, in the excretory cell of *C. elegans*. These promoter fragments are useful tools as they can be used to direct specific expression of any DNA fragment of interest in the excretory cell and excretory canal.

Accordingly, in a first aspect the invention provides a DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 2 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

The invention further provides a DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 3 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

The invention further provides a DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 4 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

The invention further provides a DNA fragment which is capable of functioning as a promoter

directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 5 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

The invention further provides a DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 6 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

The invention further provides a DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 7 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

DNA fragments having the nucleotide sequences set forth in Figures 2 to 7 exhibit tissue-specific promoter activity, directing transcription specifically in the excretory cell and excretory canal of *C. elegans*. That is to say when a reporter gene under the control of any one of these DNA fragments is introduced into *C. elegans* a high level of reporter gene expression is observed in the excretory cell and excretory canal with only background expression in other tissues. As will be described below, these tissue-specific promoters are useful tools in the construction of expression vectors which are suitable for directing gene expression specifically in the *C. elegans* excretory cell and excretory canal and in the

construction of transgenic *C. elegans* in which the transgene is expressed specifically in the excretory cell and excretory canal.

In the context of the present application, the phrase "in the absence of any further sequence of consecutive nucleotides from the *C. elegans* genome" should be interpreted as meaning in the absence of any other *C. elegans* genomic sequence consecutive with the sequences shown in Figures 2 to 7, respectively. In other words, the DNA fragments of the invention preferably contain the sequences shown in Figures 2 to 7 in the absence of any other consecutive UL6 promoter sequences.

In a second aspect the invention provides an expression vector which is suitable for directing tissue-specific expression of a heterologous DNA fragment in the excretory cell of *C. elegans*, the vector comprising a promoter comprising a DNA fragment as set forth in any one of Figures 2 to 7 positioned to direct expression of the heterologous DNA fragment.

The term "heterologous DNA fragment" refers to essentially any DNA fragment which it is desired to express in the excretory cell of *C. elegans*. This DNA fragment can be a gene, a cDNA or a fragment thereof from *C. elegans*, Drosophila sp., mouse, human, zebrafish or any other invertebrate or vertebrate origin. Alternatively, the DNA fragment may be of prokaryotic origin, a recombinant DNA or a synthetic DNA fragment. In a preferred embodiment the heterologous DNA is a reporter gene. Suitable reporter genes include those encoding green fluorescent protein (including the many GFP variants and equivalents known in the art), β-galactosidase, β-lactamase, luciferase,

acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase. The above are listed by way of example only and it is to be understood that the precise nature of the heterologous DNA fragment is not material to the invention.

In order to achieve expression in eukaryotic host cells (e.g. cells of the nematode worm *C. elegans*) an expression vector must include promoter sequences to position RNA polymerase at the transcription start site and to direct an appropriate frequency of transcription initiation at this site (e.g. to direct tissue-specific expression in the *C. elegans* excretory cell). In accordance with the invention, the promoter region of the expression vector may comprise UL6 promoter sequences which fulfill both functions (i.e. which contain the transcription initiation site for binding of RNA pol and which direct tissue-specific expression) or the promoter region of the vector may comprise a minimal promoter region from an heterologous gene (e.g. the *pes-10* promoter) which functions to position RNA polymerase at the transcription initiation site and possibly to direct a basal level of transcription and UL6 promoter sequences to direct a tissue-specific expression pattern. This will be further understood with reference to the accompanying Examples. The vector might further comprise one or more additional transcriptional regulatory elements (e.g. enhancer elements) in addition to the UL6 promoter sequences.

The expression vector may also include the following elements required for eukaryotic gene

expression: a terminator sequence and downstream polyadenylation signal for transcription termination, translation initiation sequences for ribosome binding, a start codon (usually AUG) and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or may be assembled from the elements described by methods well known in the art.

Examples of expression vectors according to the invention are plasmids, viral or phage vectors, plasmid vectors being preferred for use in *C. elegans*. Such vectors will normally possess one or more selectable markers, such as a gene for antibiotic resistance. Plasmid vectors, including those designed for expression in *C. elegans*, may also contain a bacterial origin of replication to allow replication in bacterial host cells for cloning purposes. The construction of plasmid vectors suitable for directing expression of a reporter gene in the excretory cell of *C. elegans* are described in detail in the accompanying Examples.

Also provided by the invention are host cells and organisms transformed or transfected with the expression vector.

In a still further aspect the invention provides a transgenic *C. elegans* containing a transgene comprising a promoter which is capable of directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to a protein-encoding DNA fragment.

According to the invention the transgene may comprise any promoter which is capable of directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to any DNA fragment

which it is desired to express in the excretory cell and excretory canal of *C. elegans*. Where a promoter is described herein as being capable of or suitable for directing tissue-specific gene expression in the excretory cell and/or excretory canal this should be taken to mean that the promoter directs a relatively high level of expression in the excretory cell and/or excretory canal and only background expression in other tissues. In a preferred embodiment the promoter comprises a DNA fragment, as described above, comprising a sequence of nucleotides as set forth in any one of Figures 2 to 7 in the absence of any other consecutive sequence of nucleotides from the *C. elegans* genome, i.e. in the absence of any consecutive UL6 promoter sequences. The promoter may, if appropriate, contain sequences from elsewhere in the *C. elegans* genome which are not consecutive with the sequences shown in Figures 2 to 7, for example one or more further cis-acting regulatory elements isolated from a different type of promoter.

In this context the term "transgene" refers to a DNA construct comprising a promoter operatively linked to a protein-encoding DNA fragment. The construct may contain additional DNA sequences in addition to those specified above. The transgene may, for example, form part of a plasmid vector. By the term "operatively linked" it is to be understood that the promoter is positioned to drive transcription of the protein-encoding DNA fragment.

A transgenic *C. elegans* according to this aspect of the invention may be constructed according to any of the standard techniques known to those skilled in the art. A suitable approach involves the construction

of a plasmid-based expression vector in which a protein-encoding DNA of interest is cloned downstream of a promoter capable of directing tissue-specific gene expression in the excretory cell of *C. elegans*. The plasmid vector is then injected into N2 nematodes. In order to facilitate the selection of transgenic nematodes a second plasmid carrying a dominant selectable marker may be co-injected with the experimental plasmid.

The plasmid vector is maintained in cells of the transgenic *C. elegans* in the form of an extrachromosomal array. Although plasmid vectors are relatively stable as extrachromosomal arrays they can alternatively be stably integrated into the *C. elegans* genome using standard technology, for example, using gamma ray-induced integration of extrachromosomal arrays (methods in Cell Biology, Vol 48 page 425-480).

The protein-encoding DNA fragment can be any DNA fragment which it is desired to express in the excretory canal of *C. elegans*. This DNA fragment can be a gene, a cDNA or a fragment thereof from *C.elegans*, Drosophila sp., mouse, human, zebrafish or any other invertebrate or vertebrate origin. Alternatively, the DNA fragment may be of prokaryotic origin, a recombinant DNA or a synthetic DNA fragment.

In a preferred embodiment the DNA fragment is a promoterless reporter gene encoding a marker protein such as, for example, green fluorescent protein (GFP), β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase. The expression of a marker such as GFP makes

it possible to visualize the excretory cell *in situ* in the body of the worm without intervening biochemical steps or specialized equipment such as nomarski-microscopy. A GFP-expressing excretory cell can be readily visualized using binocular microscopy following UV irradiation of the worm.

According to the invention, the transgenic *C.elegans* can be of any genetic background, for example, it can be a wild type worm, a selected mutant worm or a transgenic worm. A stably integrated transgene can easily be transferred onto a different genetic background by performing a genetic cross between a first parental *C. elegans* strain containing a stably integrated transgene and a second parental *C. elegans* strain of the desired genetic background. Standard *C. elegans* genetics can be employed for this purpose. The genetic background of the worm generally has no effect on the expression of the transgene in the excretory cell and excretory canal.

In a further embodiment of the invention the transgenic *C. elegans* further comprises a second transgene comprising a promoter which is suitable for directing tissue-specific expression in the excretory cell of *C. elegans* operatively linked to a reporter gene.

This 'double transgenic' *C. elegans* can be constructed by co-injecting *C. elegans* with two plasmid expression vectors; one containing the protein-encoding DNA fragment of interest and the other containing a reporter gene following the procedure described above. Both of the plasmid vectors can be stably integrated into the *C. elegans* genome using standard techniques (methods in Cell Biology,

Vol 48 page 425-480).

In a further aspect the invention provides a method of identifying a mutation in a gene involved in growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, the development of the excretory canal, cytoskeletal organisation, surface to cytoskeleton signalling, renal development or kidney disease, which method comprises contacting a transgenic *C. elegans* which expresses a reporter gene in the excretory canal with a mutagen and screening for phenotypic changes in the excretory canal.

Suitable mutagens for use in the method of the invention include EMS, X-rays or the UV-TMP method, all of which are known to those skilled in the art.

Following contact with mutagen the transgenic *C. elegans* are maintained in culture for at least two subsequent generations during which time observations of the morphology of the excretory canals of the progeny are made in order to identify any mutants with an abnormal excretory canal phenotype. Suitable culture conditions are described in the examples given herein.

The expression of a reporter gene such as GFP in the excretory canal allows mutations that affect the development, shape, growth direction and outgrowth of the excretory canal to be observed and selected. The affected gene is then isolated and characterized using standard genetic and molecular biology techniques.

In a preferred embodiment of the method of the invention the transgenic *C. elegans* which express a reporter gene in the excretory canal contains a

transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene. The promoter preferably comprises a DNA fragment having the sequence of nucleotides set forth in any one of Figures 2 to 7.

The method of the invention may also be adapted for use in the identifying further components of a biochemical pathway involved in growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, the development of the excretory canal, cytoskeletal organisation, surface to cytoskeleton signalling, renal development or kidney disease. In this case the mutagenesis method as described above is performed on a transgenic *C. elegans* expressing a reporter gene in the excretory cell whose genetic background is a selected mutant strain. The selected mutant strain is a strain carrying a defined mutation in a gene involved in growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, the development of the excretory canal, cytoskeletal organisation, surface to cytoskeleton signalling, renal development or kidney disease.

The selected mutant strain may be the result of a previous round of random mutagenesis performed on a wild-type *C. elegans* strain or it may be a known mutant strain, for example a knock-out mutant or an over-expressing strain taken from a *C. elegans* collection (e.g. the *C. elegans* mutant collection at the *C. elegans* Genetic Center, University of Minnesota, St Paul, Minnesota, USA). Methods for creating mutant worms with mutations in selected *C.*

elegans genes are known in the art, for example see J. Sutton and J. Hodgkin in 'The Nematode Caenorhabditis elegans' Ed. by William B. Wood and the Community of *C. elegans* Researchers CSHL, 1988 594-595; Zwaal et al. 'Target-Selected Gene Inactivation in Caenorhabditis elegans by using a Frozen Transposon Insertion Mutant Bank' 1993, Proc. Natl. Acad. Sci. USA 90 pp7431-7435; Fire et al. 'Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis elegans' 1998, Nature 391 860-811.

Further treatment of a selected mutant strain with mutagen results in the production of a double mutant but it is the phenotype of the later mutation (i.e. that resulting from contact with mutagen) which is scored by screening the subsequent generation for further morphological changes in the excretory canal. If the phenotype of the selected mutant is enhanced in the progeny after mutagenesis this indicates that the second mutation has occurred in a gene which acts on the same or a parallel biochemical pathway to the gene affected by the defined mutation. Alternatively, if the phenotype of the selected mutant is suppressed in the double-mutant progeny this indicates that the second mutation event has occurred in an important gene in the biochemical pathway.

Transgenic *C. elegans* for use in this method, or for use in any of the subsequently described methods which require the use of transgenic *C. elegans* expressing protein encoding DNAs and/or reporter genes in the excretory cell and excretory canal, may be constructed according to standard techniques known in the art such as the methodology described by Craig Mello and Andrew Fire, Methods in Cell Biology, Vol 48

Ed. H. F. Epstein and D. C. Shakes, Academic Press, pages 452-480.

The present invention further provides a method of determining whether a compound is an inhibitor or an enhancer of the regulation of growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, pathways involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling, which method comprises contacting a sample of the compound with a transgenic *C. elegans* expressing a reporter gene in the excretory canal and screening for phenotypic changes in the excretory canal.

Contact with a compound which is an inhibitor or an enhancer of the regulation of growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, pathways involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling results in changes the morphology of the excretory canal. The expression of a reporter gene in the excretory canal allows these changes in morphology to be easily visualized. Commonly observed abnormal excretory canal morphologies include: nematodes having shorter or longer canals, nematodes having curved or extra branched canals, nematodes having ventral or dorsal canals, nematodes having more or less than two canals, nematodes having wrongly branched canals, nematodes having vacuoles or cysts, nematodes with unusual features in the excretory canal.

Preferred reporter genes include those encoding green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransfersae, horseradish peroxidase, nopaline synthase or octapine synthase. In a preferred embodiment the transgenic *C. elegans* which express a reporter gene in the excretory canal contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene. The promoter preferably comprises a DNA fragment having the sequence of nucleotides set forth in any one of Figures 2 to 7. The genetic background of the transgenic worm can be wild-type, alternatively the worm can be a mutated worm or a worm expressing a second transgene.

For the avoidance of doubt, it is hereby stated that although the above-described method, and similar screening methods described hereinbelow, are based on bringing *C. elegans* worms into contact with compounds which may potentially have useful pharmacological activity there is no intention to provide any therapeutic benefit to the *C. elegans* during the screen. The worms are used merely as a tool to find out something about the properties of a compound in a biological system. In principle, this is similar to carrying out compound screening *in vitro* using mammalian cells except that the biological system is a microscopic multicellular organism rather than a single cell.

It will be appreciated that a wide variety of candidate compounds may be tested using the screening

methods described herein. The compound may be of any chemical formula and may be one of known biological or pharmacological activity, a known compound without such activity or a novel molecule such as might be present in a combinatorial library of compounds.

The invention further provides a compound which is identifiable using the above method as an inhibitor or an enhancer of the regulation of growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, pathways involved in kidney diseases, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling.

The invention also provides use of this compound as a medicament, or in the manufacture of a medicament, for promoting neuronal regeneration, re-vascularisation or wound healing or for the treatment of chronic neuro-degenerative diseases, tumour formation, metastasis, tumour vascularisation, kidney diseases, polycystic kidney diseases (specifically ADPKD), cell migration diseases or immunological diseases. Also provided by the invention is a pharmaceutical composition comprising the compound plus a pharmaceutically acceptable carrier, diluent or excipient.

The present invention further provides a method of determining the function of the protein encoded by a DNA fragment, which method comprises the steps of;

(a) providing a transgenic *C. elegans* containing a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the *C. elegans* excretory cell operatively linked to the

said DNA fragment; and

(b) observing any phenotypic changes in the excretory canal of the transgenic *C. elegans*.

According to the method of the invention the function of a given protein or peptide may be studied by expressing the protein in the excretory canal of *C. elegans*. Analysis of any resultant phenotypic changes in the excretory canal may result in a better understanding of the function of the protein.

The transgene comprises a promoter which directs transcription specifically in the *C. elegans* excretory cell and excretory canal. The DNA fragment encoding the protein of interest, which DNA fragment may be a genomic DNA, a cDNA or a fragment thereof, is placed under the control of this promoter and thereby expressed specifically in the excretory cell and excretory canal. In a preferred embodiment the promoter comprises a DNA fragment having the sequence of nucleotides set forth in any one of Figures 2 to 7.

In order to help visualise any phenotypic changes in the excretory canal the transgenic *C. elegans* may further comprise a second transgene comprising a promoter suitable for directing tissue-specific gene expression in the *C. elegans* excretory cell operatively linked to a reporter gene, preferably green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase. As described above, expression of a reporter gene such as GFP makes it possible to visualise the excretory canal without the

need for specialized equipment.

If expression of a given protein in the excretory cell results in an abnormal excretory canal phenotype then in a further embodiment the method of the invention may be combined with a mutagenesis step in order to identify further components of the biochemical pathway on which the protein acts. In this embodiment a transgenic *C. elegans* expressing the protein in the excretory canal is contacted with a mutagen, for example, EMS, UV-TMP or X-rays, all of which are well known in the art, and then maintained in culture for at least two subsequent generations during which time the morphology of the excretory canal is observed. If contact with mutagen results in either enhancement or suppression of the abnormal excretory canal phenotype then this indicates that a mutation has occurred in a gene encoding a component of the same biochemical pathway to that on which the original protein acts or of a parallel biochemical pathway. The mutated gene can then be isolated and characterised using standard molecular biology and biochemical techniques.

The invention further provides a method of determining whether a compound is a modulator of growth cone steering, cell shape, cell motility, tumour formation, metastasis, vascularisation of tumours, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling, which method comprises the steps of;

a) contacting a sample of the compound with a transgenic *C. elegans* expressing a DNA fragment

encoding a protein involved in the regulation of growth cone steering, cell shape, cell motility, renal development or a pathway involved in kidney disease, which transgenic *C. elegans* contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the *C. elegans* excretory cell operatively linked to the said DNA fragment; and

(b) screening for phenotypic changes in the excretory canal.

The transgenic *C. elegans* may be a wild type strain or a selected mutant strain. In one embodiment the transgenic worm has an abnormal excretory canal phenotype. Alternatively, the DNA fragment expressed in the excretory cell of the transgenic worm rescues an abnormal excretory canal phenotype which is present in the genetic background of the transgenic *C. elegans*.

Visible phenotypic changes in excretory canal morphology may include: nematodes having shorter or longer canals, nematodes having curved or extra branched canals, nematodes having ventral or dorsal canals, nematodes having more or less than two canals, nematodes having wrongly branched canals, nematodes having vacuoles or cysts, nematodes with unusual features in the excretory canal. To assist in visualising any phenotypic changes the transgenic worm may further express a reporter gene, preferably encoding green fluorescent protein, in the excretory canal.

The present invention further provides a compound which is identifiable as a modulator of growth cone

steering, cell shape, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling according to the above method. The invention also provides use of this compound as a medicament, or in the manufacture of a medicament, for promoting neuronal regeneration, re-vascularisation or wound healing, or for the treatment of chronic neuro-degenerative diseases, metastasis, kidney diseases, kidney cyst formation, polycystic kidney diseases (specifically ADPKD), cell migration diseases or immunological diseases. The compound may be provided as a pharmaceutical composition comprising the compound and a pharmaceutically acceptable carrier, diluent or excipient.

In a still further aspect the invention provides a method of identifying further components of a biochemical pathway on which a compound identifiable as a modulator of growth cone steering, cell shape, tumour formation, metastasis, vascularisation of tumours, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling may act, which method comprises the steps of:

(a) providing a transgenic *C. elegans* which expresses a reporter gene in the excretory canal;

(b) contacting the transgenic *C. elegans* with a mutagen;

(c) contacting the mutated *C. elegans* with a compound which is identifiable as a modulator of

growth cone steering, cell shape, tumour formation, metastasis, vascularisation of tumours, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling; and

(d) screening for phenotypic changes in the excretory canal.

Following contact with mutagen and the compound the transgenic *C. elegans* are maintained in culture for at least two subsequent generations during which time observations of the morphology of the excretory canals of the progeny are made in order to identify any changes in excretory canal phenotype. As with the other screening methods hereinbefore described the expression of a reporter gene, such as GFP, makes it easy to visualise any phenotypic changes in the excretory canal.

The compound used in this method is one which is identifiable as a modulator of growth cone steering, cell shape, tumour formation, metastasis, vascularisation of tumours, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling using one of the methods hereinbefore described for that purpose. As described above, treatment of *C. elegans* with such a compound produces morphological abnormalities in the excretory canal. Suitable mutagens for use in the method of the invention include EMS, X-rays or the UV-TMP method, all of which are known to those skilled in the art.

The use of mutagenesis in the method of the

invention facilitates the identification of further components of the biochemical pathway on which the compound acts. If the mutagenesis step results in the production of mutant progeny in which the abnormal excretory canal phenotype is enhanced (as compared with the phenotype observed following treatment of non-mutated *C. elegans* with the same compound) then the mutation has occurred in a gene in the same biochemical pathway as that on which the compound acts or a parallel pathway. Alternatively, treatment of *C. elegans* according to the method results in mutant progeny in which the abnormal excretory canal phenotype is suppressed, indicating that a mutation has occurred in a gene having an important function on the biochemical pathway on which the compound acts. In either case the gene affected by the mutation can be isolated and characterised using standard molecular biology and biochemical techniques.

In a preferred embodiment of the method of the invention the transgenic *C. elegans* which express a reporter gene in the excretory canal contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene. The promoter preferably comprises a DNA fragment having the sequence of nucleotides set forth in any one of Figures 2 to 7. The genetic background of the transgenic *C. elegans* may be wild type or it may be a selected mutant strain.

The invention will be further understood with reference to the following Examples, together with the accompanying Figures in which:

Figure 1 shows the nucleic acid sequence of the insert of pUL6#64A1.

Figure 2 shows the nucleic acid sequence of the insert of pGF2002.

Figure 3 shows the nucleic acid sequence of the insert of pGF2003.

Figure 4 shows the nucleic acid sequence of the insert of pGF2006.

Figure 5 shows the nucleic acid sequence of the insert of pGF2008.

Figure 6 shows the nucleic acid sequence of the insert of pGF2009.

Figure 7 shows the nucleic acid sequence of the insert of pGF2012.

Figure 8 shows a restriction map of the UL6 fragment.

Figure 9 is an overview of the series of plasmids containing deletion fragments of UL6.

Figure 10 is a plasmid map of pGF2006.

Figure 11 is a plasmid map of pGF2009.

Figure 12 is a plasmid map of pGF2013.

Figure 13 is a plasmid map of pGF2014.

General Experimental methods.

Sequence information and expression patterns were obtained from the *C. elegans* genome project, the Sanger Centre, and Washington University School of medicine, http/www.sanger.ac.uk/projects/C-elegans.

All Molecular biology work was performed using standard techniques known in the art, as described by Sambrook *et al.* (1989), *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press; F. M. Ausubel *et al.* (eds.) or *Current Protocols in Molecular Biology,* John Wiley & Sons, Inc. (1994), or using minor modifications of the methods described therein.

All manipulations of *C.elegans* worms were performed using techniques described in Methods in Cell Biology, vol 84; *Caenorhabditis elegans:* modern biological analysis of an organism, ed. Epstein and Shakes, academic press, 1995, or using minor modifications of the methods described therein.

Transgenic *C. elegans* strains were constructed by injection of plasmid DNA into N2 worms using standard techniques known in the art (see Methods in Cell Biology, vol 84 as mentioned above). In order to facilitate the selection of transgenic strains the plasmid pRF4 (Mello, C. C. *et al.* EMBO J. **10**, 3959-3970 (1991)) which carries the rol-6 gene was co-injected with the experimental plasmids as a marker. *C. elegans* expressing rol-6 exhibit the roller phenotype. Any other *C. elegans* dominant selectable phenotypic marker could be used in place of rol-6 with equivalent effect. When generating transgenic *C. elegans* strains with plasmids that encode for and

express GFP or GFP fusion proteins, no co-injection with a dominant selective marker is needed, as the transgenic lines can be selected by simple isolation of the progeny that expresses GFP.

Example 1

GFP expression in the excretory canal using a minimal promoter fragment.

pUL#64A1 was isolated as the result of a promoter trapping experiment described by Young J.M. and Hope I.A. Molecular markers of differentiation in *Caenorhabditis elegans* obtained by promoter trapping (1993) *Dev. Dyn.*, **196**:124-132. In this study partial Sau3AI restriction enzyme fragments of *C.elegans* genomic DNA were cloned in the *BamHI* restriction site of the vector pPD22.11 (described by Fire A, Harrison S.W., and Dixon D. A modular set of LacZ fusion vectors for studying gene expression in *Caenorhabditis elegans* (1990) *Gene* **93**:189-198.) creating LacZ translational fusions. Introduction of pUL#64A1 into *C.elegans* resulted in the expression of β-galactosidase in the excretory cell and excretory canal and the lateral nuclei of the hypodermis adjacent to the anterior and posterior of the excretory cell. The region of the genomic DNA insert immediately adjacent to the lacZ gene was sequenced enabling the origin of the insert of pUL#64A1 to be localized on the *C. elegans* physical genome map.

In order to determine the length of the cloned insert the vector pUL#64A1 was digested with several restriction enzymes, including *Xho*I, *Sal*I, *Sph*I and *Hind*III. Restriction fragment length analysis showed

that the cloned fragment in pUL#64A1 was approximately 6 kb in length. Analysis of the genomic DNA of *C.elegans* (available from the *C.elegans* genome project, the Sanger Centre, and Washington University School of medicine, http/www.sanger.ac.uk/projects/C-elegans) revealed the presence of two adjacent *Sau*3AI in the region of the pUL#64A1 insert. This confirmed that the inserted DNA fragment had a length of 6065 bp or 6023 bp and could be located between positions 33561 and 39620 on cosmid C17H12. The inserted DNA fragment was designated UL6.

Analysis of the nucleotide sequence of the UL6 insert revealed the presence of two putative genes orientated in opposite directions. The putative promoter located upstream of the gene orientated in the same direction as the lacZ reporter gene was considered to be the most relevant for further analysis.

In order to test for promoter activity several deletion fragments of UL6 spanning this region were cloned in the GFP-fusion vector pPD95.79 (constructed by Andrew Fire, Carnegie Institute of Washington and freely available at ftp://stein.cshl.org/pub/elegans_vector/). The promoter activity of each of the fragments was then tested by injecting the plasmids into *C. elegans* and analysing the levels of GFP expression in different tissues.

A first construct, designated pGF2002, contained the 3.2 kb *Hind*III-*Msc*I fragment of UL6. Following injection of pGF2002 into *C. elegans* worms, the F1 generation showed GFP expression in the head, tail, muscles and excretory canal, whilst the F2 generation (and subsequent generations) expressed GFP in the

excretory canal with background expression in other cells. The background expression in the other cells is mosaic and depending from animal to animal the pharynx, the gut, muscle cells or the tail may show background GFP expression. The resulting strain was designated UG266 (bgEx34).

A smaller construct containing a 2.2 kb *SphI-Msc*I fragment of UL6 in pPD95.79 was made and designated pGF2003. Following injection of pGF2003 into *C.elegans* worms, the F1 showed GFP expression in the head, tail, muscles and excretory canal, analogous to pGF2002. The expression of GFP in the F2 generation was not analysed but is expected to be analogous to that observed with pGF2002, i.e. strong expression in the excretory canal with background expression in other tissues.

Finally, a 0.9 kb *Nru*I-*Msc*I fragment of UL6 was cloned into pPD95.79 digested with *MscI* and *Xba*I, the later made blunt with Klenow polymerase. After injection of the resultant plasmid, designated pGF2001, into *C. elegans* no GFP expression could be observed.

These results and the fact that the excretory cell-specific promoter in the UL6 fragment was thought to be localized between the two genes, being approximately between the *Nru*I and the *Sph*I site, stimulated the inventors to analyse further deletion constructs. The aim was to determine the smallest fragment of UL6 that promotes the transcription and expression in the *C. elegans* excretory cell and excretory canal.

The 1.3 kb *Nru*I-*Sph*I insert of pGF2003 was excised and cloned into the vector pPD97.78. The

latter vector is analogous to pPD95.79, but contains a minimal *pes-10* promoter upstream of the GFP gene. This means that it is not necessary to make a fusion construct between the fragment to be tested for promoter activity and GFP. Vector pPD97.78 was digested with *Sph*I and *Hin*dII and the resulting plasmid was designated pGF2006. Transgenic *C. elegans* animals harboring this plasmid from the F2 and subsequent generations show strong GFP expression in the excretory canal with only minor background expression in other cell types. The new *C. elegans* strain was designated UG267(bgEx35).

A 1.7 kb MscI-SacI fragment of pGF2003 was cloned in pPD95.79 and designated pGF2008. After injection of this plasmid into *C. elegans* worms the F1 generation showed GFP expression in the excretory canal, gut, head, tail and spermatheca.

The region that promotes the expression of GFP in the excretory canal seemed to be located in the 1.3 kb *Nru*I-*Sph*I insert of pGF2006. Further deletion analysis was therefore carried out to identify the minimal UL6 promoter in this fragment. Plasmid pGF2009 was generated by deleting a 0.5 kb *Bpi*I-*Hind*III fragment of pGF2006, leaving a 0.9 kb fragment. The *Hind*III site is part of the multiple cloning site of the vector and adjacent to the *Sph*I site. Plasmid pGF2010 was generated by deleting a 0.9bp *Xba*I-*Bpi*I fragment of pGF2006, leaving a 0.5 kb fragment. The *Xba*I site is part of the multiple cloning site of the vector and adjacent to the *Nru*I-*Hind*II fusion. The construction of this series of deletions will be further understood with reference to Figure 8 which shows a restriction map of the UL6 insert and Figure 9 which gives an

overview of the UL6 deletion constructs.

C. elegans injected with pGF2009 showed GFP expression in the excretory canal, with only minor background expression in the other cells, analogous to the expression results with pGF2006. The resulting *C. elegans* strain was designated UG271 (bgEX38). No GFP could be detected in the F1 generation after introduction of pGF2010 into *C. elegans*.

Two further deletions have been constructed to define the minimal promoter capable of directing transcription in the excretory canal. pGF2009 was digested with restriction enzyme *Bsa*I and made blunt with klenow polymerase. The resulting fragment was further digested with *Xba*I to give a 0.4 kb *Bsa*I-*Xba*I fragment and with *Hind*III to give a 0.5 kb *Hind*III-*Bsa*I fragment. These fragments were independently cloned in pPD97.78, using the *Hind*III-*Stu*I and *Stu*I-*Xba*I sites, respectively. The resulting vectors are designated pGF2011 and pGF2012. Injection of pGF2012 into *C. elegans* resulted in expression of GFP in the excretory canal.

Plasmid expression vectors suitable for expressing GFP or lacZ or any other reporter protein in the excretory canal of *C. elegans* can be integrated in the genome of the worm using standard technology . The vectors pGF2006, pGF2009 and pGF2012 are particularly suitable for this purpose.

Example 2

Methods of screening for new mutations.

To develop a screen for new mutants having a

"short canals" phenotype a *C. elegans* strain which contains any of the above plasmids that express GFP in the excretory canal can be used. To develop a screen for new mutants with a "ventral canals" phenotype a worm strain with a stably integrated GFP-expressing plasmid is crossed with a *C. elegans* strain having the UNC phenotype, such as *C. elegans* strain MT152, *unc-53* (n152) (obtained from Dr Bob Horvitz MIT, Cambridge MA, USA). F2 worms with short canals (UNC phenotype) are used for further screening.

General protocol for mutagenesis.

A few thousand adult worms of the desired genetic background are treated with a hypochlorite solution to get a synchronised culture. This culture is then mutagenized using the EMS technique when the worms have reached the L4 stage (protocol for mutagenesis is as described in "Methods in Cell Biology, Vol 48 page 31-35"). As an alternative to EMS the UV-TMP technique can be used. In this method the worms are contacted with tri-methyl-psoralen and then treated with UV radiation, as described in Methods in Cell Biology, Vol 48. *Caenorhabditis elegans*: Modern biological analysis of an organism. Eds H. F. Epstein and D. C. Shakes, Academic Press. F2 worms are analysed for aberrant excretory canal phenotypes, which can easily be visualised due to the expression of GFP.

Observations of excretory canal morphology are made for two subsequent generations following contact with mutagen. Dependent on the starting genetic background of the worms, the following defects can be observed in the progeny:- nematodes having shorter or

longer canals, nematodes having curved or extra branched canals, nematodes having ventral or dorsal canals, nematodes having more less than two canals, nematodes having wrongly branched canals , nematodes having vacuoles or cysts, nematodes with unusual features in the excretory canal.

Example 3

A method of detecting compounds that influence the excretory canal phenotype.

Worms stably expressing GFP in the excretory canal can be used to detect and isolate compounds that have effects on the morphology of the excretory canal as follows:

Standard agar plates for use with *C. elegans* are seeded with *E.coli* and left to full growth. Serial dilutions of compound to be tested are then pipetted onto the *E.coli* lawn and allowed to diffuse into the lawn. One transgenic *C. elegans* expressing GFP in the excretory canal (L4 stage) per dilution of compound is put onto the bacterial lawn. The plates are incubated at 21°C and visually screened for excretory canal morphology at various time intervals and for two generations to detect aberrant phenotypes. The following defects in excretory canal morphology can be observed; nematodes having shorter or longer canals, nematodes having curved or extra branched canals, nematodes having ventral or dorsal canals, nematodes having more or less than two canals, nematodes having wrongly branched canals, nematodes having vacuoles or cysts, nematodes with unusual features in the

excretory canal.

Example 4

Use of the UL6 minimal promoter fragment in expression of a heterologous DNA in the excretory canal.

The above-described plasmids pGF2002, pGF2003, pGF2006, pGF2008, pGF2009 and pGF2012 have all been used to express GFP in the excretory cell and excretory canal of *C.elegans*.

The same plasmids can be used to drive the transcription of other DNA fragments than the GFP encoding fragment by simply replacing this GFP encoding fragment with any other DNA fragment of interest. Preferentially the vectors pGF2006, pGF2009 or pGF20012 are used for this purpose. As an alternative any of the isolated and analysed promoter fragments described above and shown in Figures 2-7 can be cloned upstream of the target DNA of interest in an expression vector suitable for use in *C. elegans*.

Example 5

Compound screening assay.

The following method may be used to determine whether a compound is a modulator of growth cone steering, cell shape, cell motility, tumour formation, tumour vascularisation, metastasis, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling:

Standard agar plates for use with *C. elegans* are

seeded with *E.coli* and left to full growth. Serial dilutions of compound to be tested are then pipetted onto the *E.coli* lawn and allowed to diffuse into the lawn. One transgenic *C. elegans* (L4 stage) per dilution of compound is put onto the bacterial lawn. The transgenic *C. elegans* is one expressing both a protein involved in the regulation of growth cone steering, cell shape, cell motility, tumour formation, tumour vascularisation, metastasis, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling and GFP in the excretory canal. The plates are incubated at 21°C and visually screened for excretory canal morphology at various time intervals and for two generations to detect aberrant phenotypes.

The following abnormal excretory canal morphologies can be observed; nematodes having shorter or longer canals, nematodes having curved or extra branched canals, nematodes having ventral or dorsal canals, nematodes having more or less than two canals, nematodes having wrongly branched canals nematodes having vacuoles or cysts, nematodes with unusual features in the excretory canal.

Example 6

Construction of a *C. elegans* strain stably expressing a reporter gene in the excretory canal.

Although expression of reporter genes in the excretory canal from extrachromosomal arrays is sufficient to perform most applications, stable

expression in *C. elegans* by integration would facilitate and improve most if not all the applications. Several *C. elegans* strains have been constructed, wherein the DNA encoding for the promoter (here designated as UL6) that drives the expression of the reporter gene, in this case GFP, is integrated the in the genome.

Method

A general method to integrate extrachromosomal DNA in to the genome of *C. elegans* has been described by Mello and Fire in Methods in Cell biology, Volume 48, *Caenorhabditis elegans*: Modern biological analysis of an organism, Chapter 19, 466-468. Here the construction of a particular example is described in detail:

A wild type N2 *C. elegans* strain was injected with pGF2006 (100ng/µl) and with pUC18DNA (100ng/µl) using standard methods, resulting in strain UG489 *bgEx176*. Although a relatively high transmission efficiency (up to 55%) was observed, which troubles later selections, a clean expression of GFP was observed in the excretory cell and the excretory canal.

9cm NGM plates with a population of late stage L4 worms were gamma-irradiated for 1 hour in order to obtain a total intensity of 30 gray. 6 x 35 animals were isolated after irradiation, incubated for 24 hours at 20°C and then transferred to fresh plates and further incubated at 25°C. 2 x 500 F1 animals were isolated after 48 hours and after 72 hours respectively, and further incubated at 25°C. 2-4 F2's per F1 were isolated and incubated at 15°C.

The progeny of these F2's was checked by selecting for plates with 75% or 100% of the progeny expressing GFP. Of these positive plates, 4 F3's were isolated and further incubated at 15°C. Again the progeny was checked for the presence of 75% to 100% of the worms expressing GFP.

The resulting integrated lines were crossed out several times to confirm that no major translocations or unlinked mutations have occurred as a result of the radiation treatment. This was done by mating wild-type *C. elegans* (N2) males with the hermaphrodites carrying the integration (the N2 strain can be obtained from CGC, University of Minnesota, USA). F1 males are used to cross to the mapping strains and to cross back to N2 hermaphrodites (crossing out once every generation by always using GFP male progeny, except if the site of the integration is on X).

The integrations were mapped to the *C. elegans* chromosomes, by applying standard techniques well known in the art. Mapping was performed using at least following strains: MSI dpy-5(e61)I;unc-4(e120)II; lon-1(e185)III and MT464 unc-5(e53)IV; dpy-11(e224)V; lon-2(e678)X.

Result

Of a total of 1000 F1 worms primary isolated, 650 lines were further examined. Out of these examined lines, eight integrated lines were retained, crossed out and mapped:

UG703 bgIs309 has strong hypodermal GFP expression, integrated on X;

UG704 bgIs310 has low hypodermal GFP expression, integrated on X;

UG705 bgIs311 has nearly no hypodermal GFP expression, integrated on X;
UG706 bgIs312 has nearly no hypodermal GFP expression, integrated on I;
UG707 bgIs313 has strong hypodermal GFP expression, integrated on IV;
UG708 bgIs314 has nearly no hypodermal GFP expression, integrated on X;
UG709 bgIs315 has low hypodermal GFP expression, integrated on X;
UG710 bgIs316 has low hypodermal GFP expression, integrated on V.

Conclusion

Of the eight independent integrated lines obtained, at least five are of good quality (bgIs311, bgIs312, bgIs314, bgIs315 and bgIs316).

Example 7

Construction of improved vectors to express genes in the *C. elegans* excretory cell.

The plasmid expression vectors pGF2006 and pGF2009 for expression of the reporter gene GFP both contain a nuclear localisation signal (NLS) in fusion with the reporter gene. Expression of the fusion protein (NLS-GFP) results in the translocation of a part of the total amount of the protein expressed to the nucleus of the excretory cell. Although the expression of the fusion protein is high enough to visualise the fusion protein in the whole excretory cell including the excretory canal, the present inventors decided to delete this NLS part. Expression

from the UL6 promoter would hence no more result in translocation in the nucleus of the expressed gene, and provide for a more equal localisation of the expressed gene.

To test this hypothesis the NLS signal was deleted from the plasmids pGF2006 and pG2009. This was easily done by deletion of a KpnI restriction fragment resulting in the plasmids pGF2013 and pGF2014 respectively.

SEQUENCE LISTING

SEQ ID NO: 1 is the nucleotide sequence of the insert of pUL6#64A1, illustrated in Figure 1.

SEQ ID NO: 2 is the nucleotide sequence of the insert of pGF2002, illustrated in Figure 2.

SEQ ID NO: 3 is the nucleotide sequence of the insert of pGF2003, illustrated in Figure 3.

SEQ ID NO: 4 is the nucleotide sequence of the insert of pGF2006, illustrated in Figure 4.

SEQ ID NO: 5 is the nucleotide sequence of the insert of pGF2008, illustrated in Figure 5.

SEQ ID NO: 6 is nucleotide sequence of the insert of pGF2009, illustrated in Figure 6.

SEQ ID NO: 7 is the nucleotide sequence of the insert of pGF2012, illustrated in Figure 7.

SEQ ID NO: 8 is the complete nucleotide sequence of plasmid pGF2006.

SEQ ID NO: 9 is the complete nucleotide sequence of plasmid pGF2009.

SEQ ID NO: 10 is the complete nucleotide sequence of plasmid pGF2013.

SEQ ID NO: 11 is the complete nucleotide sequence of plasmid pGF2014.

The contents of any published patents, patent applications and scientific publications referred to herein are to be incorporated herein by reference.

Claims:

1. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 2 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

2. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 3 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

3. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 4 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

4. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 5 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

5. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 6 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

6. A DNA fragment which is capable of functioning as a promoter directing gene expression in the excretory cell of *C. elegans*, which DNA fragment comprises the sequence of nucleotides set forth in Figure 7 or a fragment thereof in the absence of any other sequence of consecutive nucleotides from the *C. elegans* genome.

7. An expression vector which is suitable for directing tissue-specific expression of a heterologous DNA fragment in the excretory cell of *C. elegans*, said vector comprising a promoter comprising a DNA fragment as claimed in any one of claims 1 to 6 positioned to direct expression of the said heterologous DNA fragment.

8. An expression vector as claimed in claim 7 wherein the heterologous DNA fragment is a reporter gene.

9. An expression vector as claimed in claim 8 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase,

horseradish peroxidase, nopaline synthase or octapine synthase.

10. A host cell transformed or transfected with an expression vector as claimed in any one of claims 7 to 9.

11. A transgenic *C. elegans* containing a transgene comprising a promoter which is capable of directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to a protein-encoding DNA fragment.

12. A transgenic *C. elegans* as claimed in claim 11 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

13. A transgenic *C. elegans* as claimed in claim 11 or claim 12 wherein the protein-encoding DNA fragment comprises a reporter gene encoding green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

14. A transgenic *C. elegans* as claimed in any one of claims 11 to 13 wherein said transgene is stably integrated into a chromosome of the said *C. elegans*.

15. A transgenic *C. elegans* as claimed in claim 11 or claim 12 which further comprises a second

transgene, which transgene comprises a promoter suitable for directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to a reporter gene.

16. A transgenic *C. elegans* as claimed in claim 15 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

17. A transgenic *C. elegans* as claimed in claim 15 or claim 16 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

18. A transgenic *C. elegans* as claimed in any one of claims 15 to 17 wherein one or both of the transgenes is integrated into the chromosome of the said *C. elegans*.

19. A method of identifying a mutation in a gene involved in growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, kidney disease, the development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling, which method comprises contacting a transgenic *C. elegans* which expresses a reporter gene in the excretory canal with a mutagen and screening for phenotypic changes in the excretory canal.

20. A method as claimed in claim 19 wherein the mutagen is EMS, UV-TMP or X-rays.

21. A method as claimed in claim 19 or claim 20 wherein the transgenic *C. elegans* contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene.

22. A method as claimed in claim 21 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

23. A method as claimed in any one of claims 19 to 22 wherein the transgenic *C. elegans* is a wild-type strain or a selected mutant strain.

24. A method as claimed in any one of claims 19 to 23 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

25. A method of determining whether a compound is an inhibitor or an enhancer of the regulation of growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, pathways involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling, which method comprises contacting a sample

of the compound with a transgenic *C. elegans* which expresses a reporter gene in the excretory canal and screening for phenotypic changes in the excretory canal.

26. A method as claimed in claim 25 wherein said transgenic *C. elegans* is a wild-type strain or a selected mutant strain.

27. A method as claimed in claim 25 or claim 26 wherein the transgenic *C. elegans* contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene.

28. A method as claimed in claim 27 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

29. A method as claimed in any one of claims 25 to 28 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

30. A compound which is identifiable as an inhibitor or an enhancer of the regulation of growth cone steering, cell motility, cell shape, tumour formation, metastasis, vascularisation of tumours, renal development, pathways involved in kidney disease, development of the excretory canal,

cytoskeletal organisation or surface to cytoskeleton signalling using the method of any one of claims 25 to 29.

31. A compound as claimed in claim 30 for use as a medicament for promoting neuronal regeneration, re-vascularisation or wound healing.

32. A compound as claimed in claim 30 for use as a medicament for the treatment of metastasis, tumour formation, tumour vascularisation, chronic neuro-degeneration, kidney disease, kidney cyst formation, polycystic kidney diseases, cell migration diseases or immunological diseases.

33. Use of a compound as claimed in claim 30 in the manufacture of a medicament for promoting neuronal regeneration, re-vascularisation or wound healing.

34. Use of a compound as claimed in claim 30 in the manufacture of a medicament for the treatment of metastasis, kidney disease, kidney cyst formation, polycystic kidney diseases, cell migration diseases or immunological diseases.

35. A pharmaceutical composition comprising a compound as claimed in any one of claims 30 to 34 and a pharmaceutically acceptable carrier, diluent or excipient therefor.

36. A method of determining the function of the protein encoded by a DNA fragment, which method comprises the steps of;

(a) providing a transgenic *C. elegans* containing a transgene comprising a promoter suitable for directing tissue-specific gene expression in the *C. elegans* excretory cell operatively linked to the said DNA fragment; and

(b) observing any phenotypic changes in the excretory canal of the transgenic *C. elegans*.

37. A method as claimed in claim 35 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

38. A method as claimed in claim 36 or claim 37 wherein the transgenic *C. elegans* further comprises a second transgene comprising a promoter suitable for directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to a reporter gene.

39. A method as claimed in claim 38 wherein the promoter of the second transgene comprises a DNA fragment as claimed in any one of claims 1 to 6.

40. A method as claimed in claim 38 or claim 39 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

41. A method of determining whether a compound is a modulator of growth cone steering, cell shape,

tumour formation, metastasis, vascularisation of tumours, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling, which method comprises the steps of;

(a) contacting a sample of the compound with a transgenic *C. elegans* expressing a DNA fragment encoding a protein involved in the regulation of growth cone steering, cell shape, cell motility, renal development or a pathway involved in kidney disease, which transgenic *C. elegans* contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the *C. elegans* excretory cell operatively linked to the said DNA fragment; and

(b) screening for phenotypic changes in the excretory canal.

42. A method as claimed in claim 41, wherein said transgenic *C. elegans* is a wild-type strain or a selected mutant strain.

43. A method as claimed in claim 41 or claim 42 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

44. A method as claimed in any one of claims 41 to 43 wherein the transgenic *C. elegans* further contains a second transgene comprising a promoter suitable for directing tissue-specific gene expression in the excretory cell of *C. elegans* operatively linked to a reporter gene.

45. A method as claimed in claim 44 wherein the promoter of the second transgene comprises a DNA fragment as claimed in any one of claims 1 to 6.

46. A method as claimed in claim 44 or claim 45 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, β-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase or octapine synthase.

47. A method as claimed in any one of claims 41 to 46 wherein the transgenic *C. elegans* has an abnormal excretory canal phenotype.

48. A method as claimed in any one of claims 41 to 47 wherein the DNA fragment expressed in the excretory cell of the transgenic *C. elegans* rescues an abnormal excretory canal phenotype which is present in the genetic background of the transgenic *C. elegans*.

49. A compound which is identifiable as a modulator of growth cone steering, cell shape, cell motility, tumour formation, metastasis, vascularisation of tumours, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling using the method of any one of claims 41 to 49.

50. A compound as claimed in claim 49 for use as a medicament for promoting neuronal regeneration, re-

vascularisation or wound healing.

51. A compound as claimed in claim 49 for use as a medicament for the treatment of chronic neuro-degenerative diseases, metastasis, tumour formation, tumour vascularisation, kidney diseases, kidney cyst formation, polycystic kidney diseases, cell migration diseases or immunological diseases.

52. Use of a compound as claimed in claim 49 in the manufacture of a medicament for promoting neuronal regeneration, re-vascularisation or wound healing.

53. Use of a compound as claimed in claim 49 in the manufacture of a medicament for the treatment of chronic neuro-degenerative diseases, metastasis, tumour formation, tumour vascularisation, kidney diseases, kidney cyst formation, polycystic kidney diseases, cell migration diseases or immunological diseases.

54. A pharmaceutical composition comprising a compound as claimed in any one of claims 49 to 53 and a pharmaceutically acceptable carrier, diluent or excipient therefor.

55. A method of identifying further components of a biochemical pathway on which a compound identifiable as a modulator of growth cone steering, cell shape, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling may act, which method

comprises the steps of:

(a) providing a transgenic *C. elegans* which expresses a reporter gene in the excretory canal;

(b) contacting the transgenic *C. elegans* with a mutagen;

(c) contacting the mutated *C. elegans* with a compound which is identifiable as a modulator of growth cone steering, cell shape, cell motility, renal development, a pathway involved in kidney disease, development of the excretory canal, cytoskeletal organisation or surface to cytoskeleton signalling; and

(d) screening for phenotypic changes in the excretory canal.

56. A method as claimed in claim 55 wherein the mutagen is EMS, UV-TMP or X-rays.

57. A method as claimed in claim 55 or claim 56 wherein the transgenic *C. elegans* contains a transgene comprising a promoter which is suitable for directing tissue-specific gene expression in the excretory canal of *C. elegans* operatively linked to a reporter gene.

58. A method as claimed in claim 57 wherein the promoter comprises a DNA fragment as claimed in any one of claims 1 to 6.

59. A method as claimed in any one of claims 55 to 58 wherein the reporter gene encodes green fluorescent protein, β-galactosidase, β-lactamase or luciferase.

FIG. 1.

gatcgctcgaataaaaatttataatgtcagattacgttttagatccaaaaaa
attaggctgaatccgttttgattctctagaattttgtttagccaaaaaacatcg
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FIG. 1.(CONTINUED)

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FIG. 1. (CONTINUED)

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FIG. 2.

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FIG. 2. (CONTINUED)

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FIG. 3.

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FIG. 4.

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FIG. 5.

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FIG. 6.

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FIG. 7.

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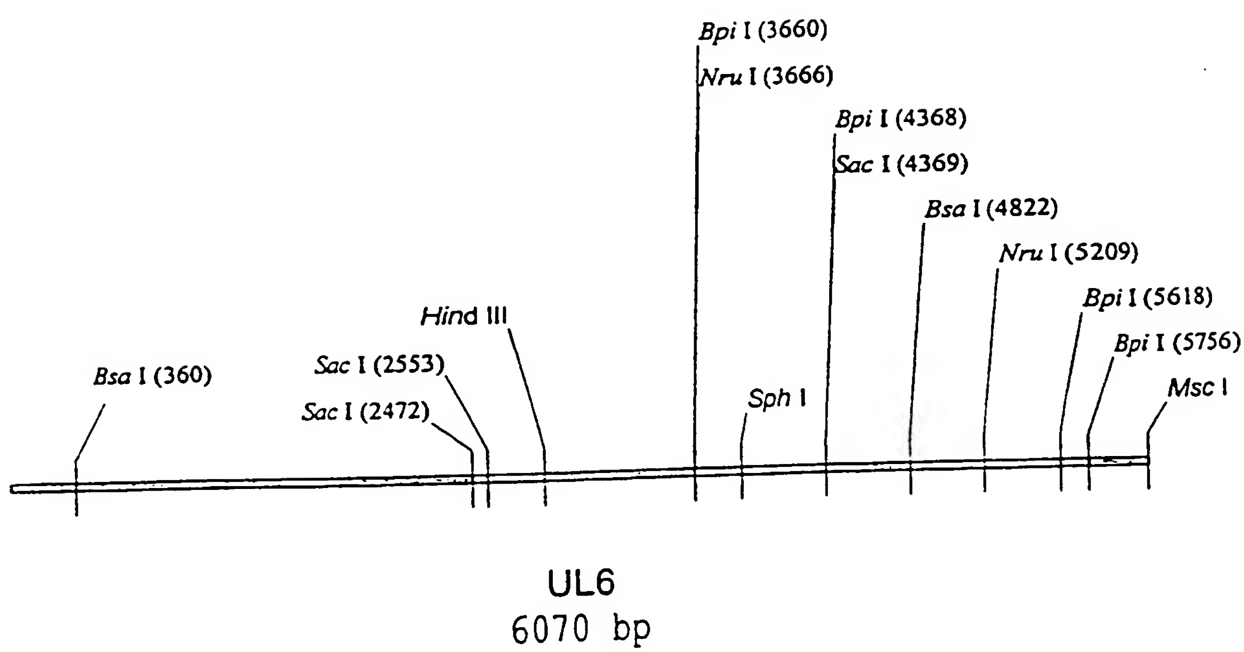
FIG. 8.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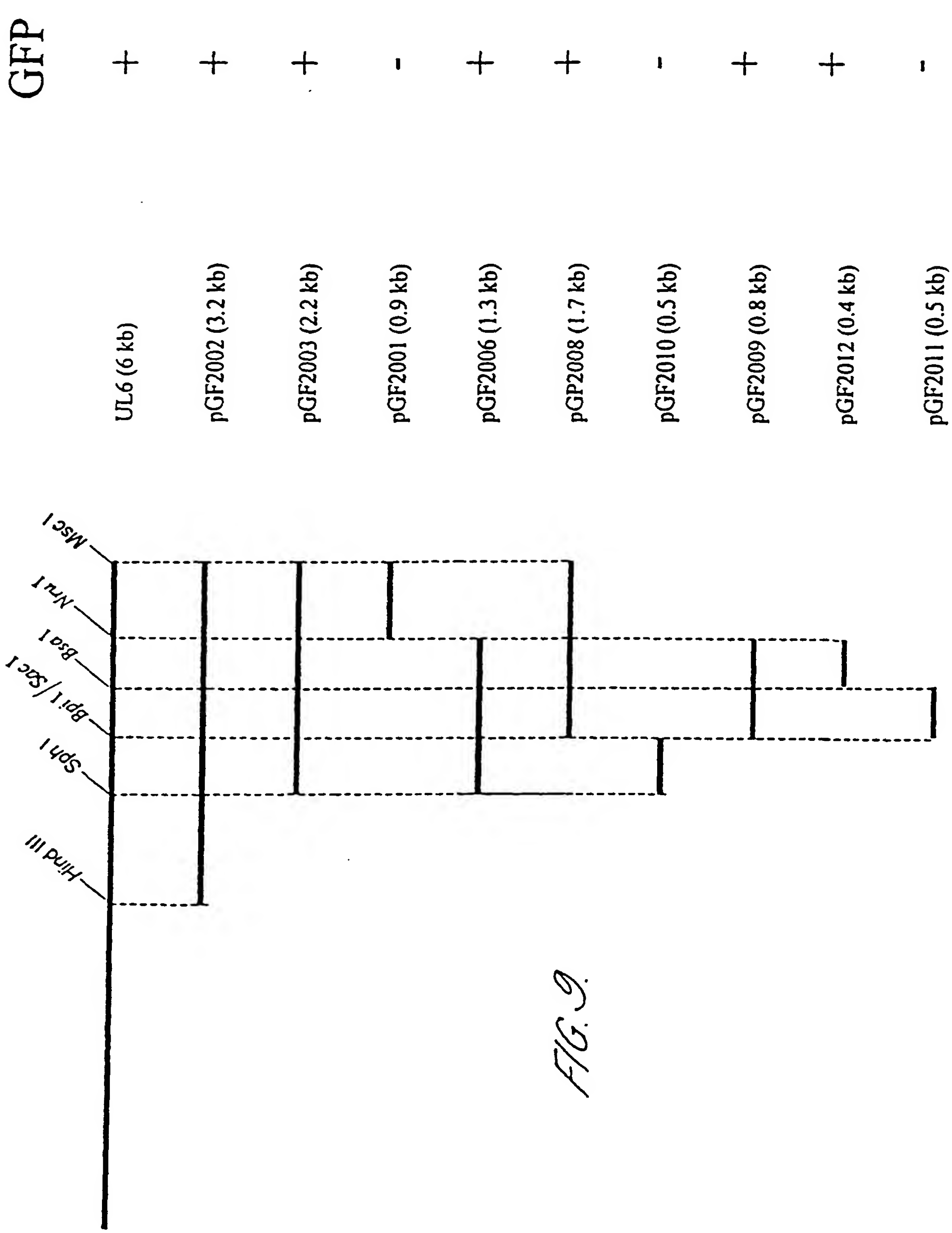
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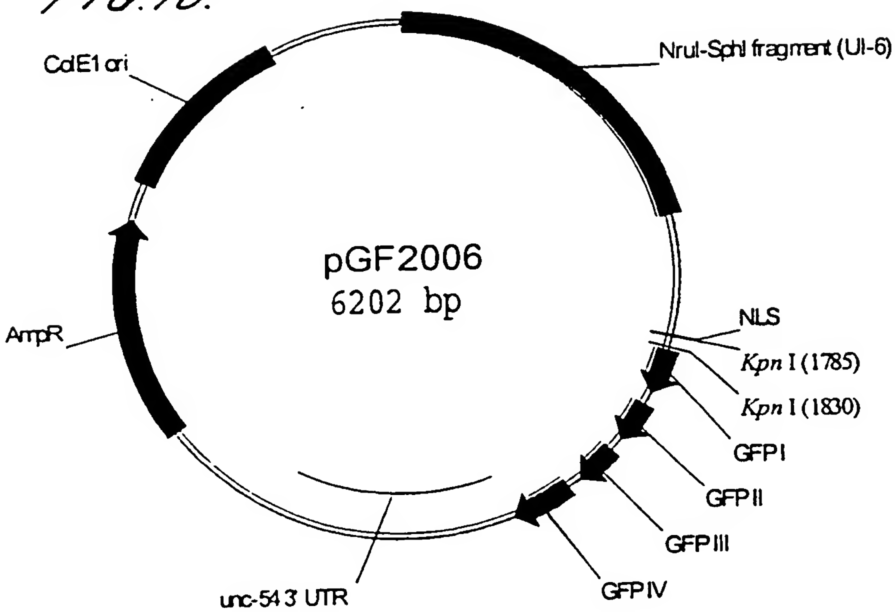
FIG. 9.

FIG. 10.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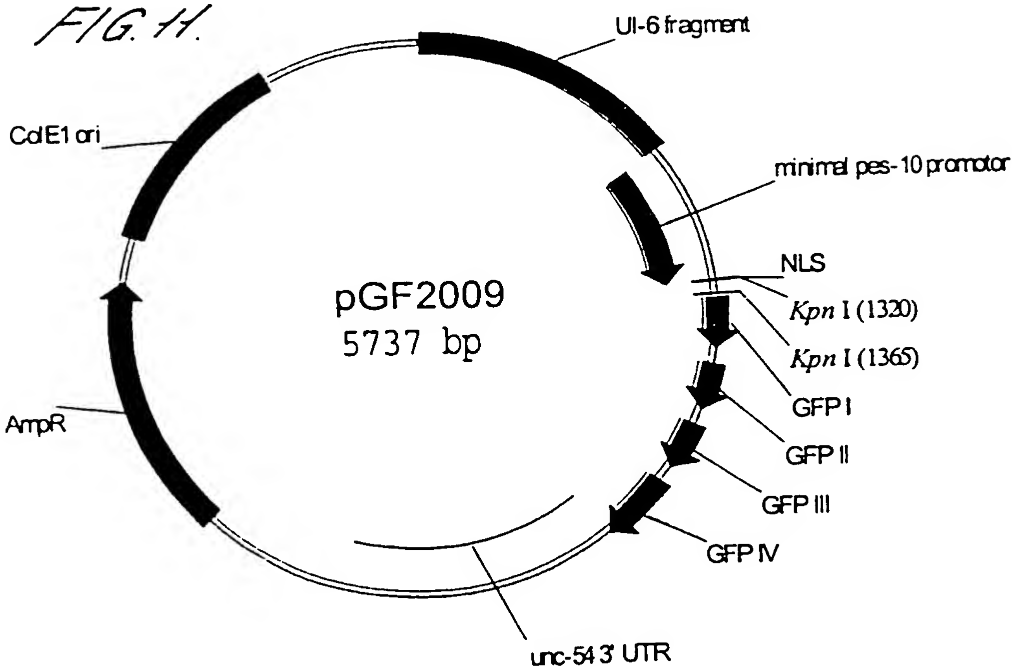
GFP III
GFP IV
unc-54 3' UTR

FIG. 11.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minimal pes-10 promotor
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unc-54 3' UTR

FIG. 12.

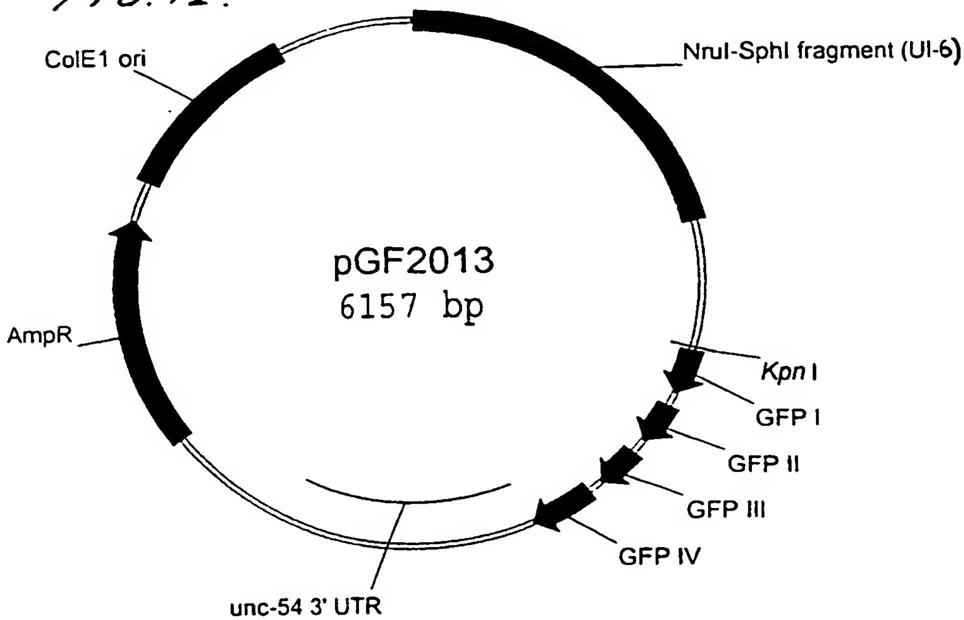
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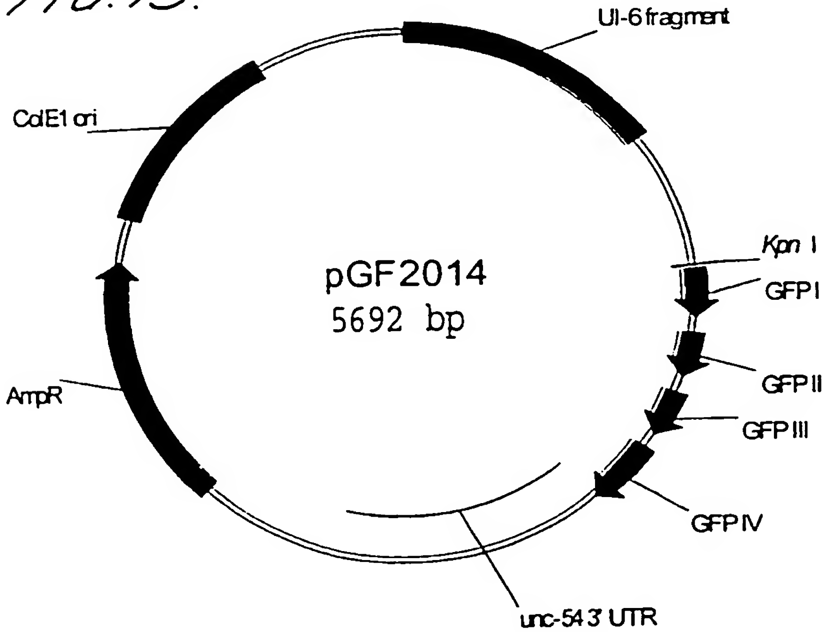
FIG. 13.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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organizati n
International Bureau

(43) International Publication Date
21 September 2000 (21.09.2000)

PCT

(10) International Publication Number
WO 00/54815 A3

(51) International Patent Classification[7]: C12N 15/12, 15/85, 5/10, C07K 14/435, A01K 67/033, C12Q 1/68, A61K 49/00

(21) International Application Number: PCT/EP00/02373

(22) International Filing Date: 16 March 2000 (16.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9906018.8 16 March 1999 (16.03.1999) GB

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(75) Inventors/Applicants (*for US only*): ZWAAL, Richard [BE/BE]; Devgen NV, Technologiepark 9, B-9052 Zwijnaarde (BE). ASAERT, Wouter [BE/BE]; Devgen NV, Technologiepark 9, B-9052 Zwijnaarde (BE). ROELENS, Ingele [BE/BE]; Devgen NV, Technologiepark 9, B-9052 Zwijnaarde (BE). BOGAERT, Thierry [BE/BE]; Devgen NV, Technologiepark 9, B-9052 Zwijnaarde (BE).

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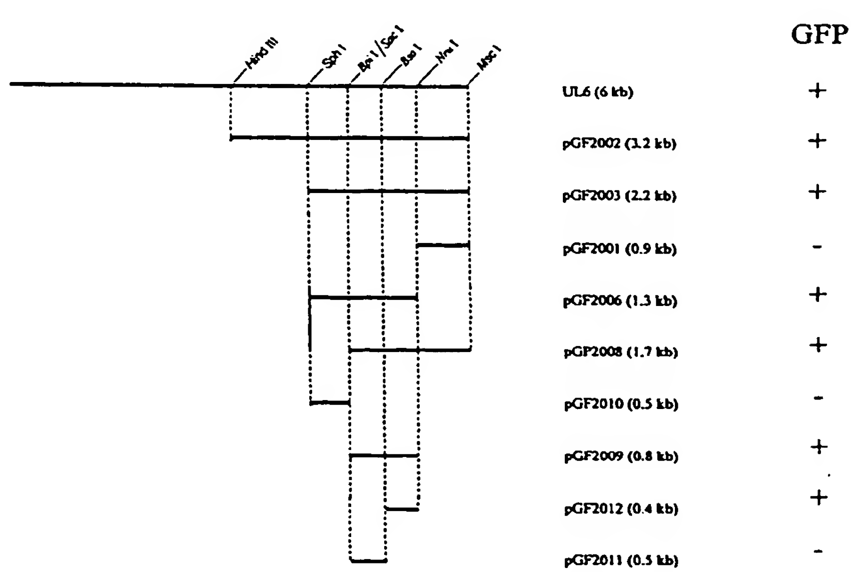
(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: EXPRESSION OF DNA OR PROTEINS IN *C. ELEGANS*



(57) Abstract: DNA fragments from the promoter region of the *C. elegans* UL6 gene which are capable of functioning as promoters directing gene expression in the excretory cell of *C. elegans* are provided and also expression vectors and transgenic *C. elegans* containing these fragments. Also provided are screening methods performed in *C. elegans* for identifying compounds or mutations which have an effect on the morphology of the excretory canal. Compounds identified using these screening methods may have therapeutic potential in the treatment of a range of diseases for which the *C. elegans* excretory canal serves as a model.

WO 00/54815 A3

Published:
— *With international search report.*

(88) Date of publication of the international search report: 25 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02373

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/85 C12N5/10 C07K14/435 A01K67/033
C12Q1/68 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOUNG J AND HOPE I: "Molecular markers of differentiation in Caenorhabditis elegans obtained by promoter trapping." DEV DYN., vol. 196, no. 2, February 1993 (1993-02), pages 124-132, XP002146202 cited in the application page 128, column 2, paragraph 6 -page 129, column 1, paragraph 4; table 2	1-14
X	BROEKS A ET AL: "A P-glycoprotein protects Caenorhabditis elegans against natural toxins" EMBO JOURNAL., vol. 14, no. 9, 1 May 1995 (1995-05-01), pages 1858-1866, XP002146203 figures 3-5	11,13, 25,26, 36, 41-43, 47,48

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 2000	15/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

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Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/EP 00/02373

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAMBIE E ET AL: "Two homologous regulatory genes, lin-12 and glp-1, have overlapping functions" DEVELOPMENT, vol. 112, no. 1, May 1991 (1991-05), pages 231-240, XP000938910 page 235, column 2, paragraph 2 -page 238, column 1, paragraph 1	19,20, 23,55,56
A	C. ELEGANS SEQUENCING CONSORTIUM: "Genome sequence of the nematode C. elegans: a platform for investigating biology. The C. elegans Sequencing Consortium" SCIENCE., vol. 282, no. 5396, 11 December 1998 (1998-12-11), pages 2012-2018, XP002146204 cited in the application	1-6
A	-& DATABASE EM-INV E.B.I., Hinxton, U.K.; Accession Number: AF045642, 11 February 1998 (1998-02-11) GEISEL C: "The sequence of C. elegans cosmid C17H12" XP002146205 Nucleotides 10576-13785: 100% identity in 3210 nt overlap with SeqIdNo.2 abstract	
A	NELSON F AND RIDDLE D: "Functional study of the Caenorhabditis elegans secretory-excretory system using laser microsurgery" J EXP ZOOL, vol. 231, no. 1, July 1984 (1984-07), pages 45-56, XP000938887	
A	WO 97 11956 A (UNIV COLUMBIA ;GREENWALD IVA (US); LEVITAN DIANE (US)) 3 April 1997 (1997-04-03) page 18, line 26-29	
A	WO 98 28971 A (LINK CHRISTOPHER ;UNIV TECHNOLOGY CORP (US)) 9 July 1998 (1998-07-09)	
A	WO 96 38555 A (BOGAERT THIERRY ;STRINGHAM EVE (CA); VANDEKERCKHOVE JOEL (BE)) 5 December 1996 (1996-12-05) page 57, line 17 - line 20	
A	WO 98 24810 A (BOGAERT THIERRY ANDRE OLIVIER ;DERAEYMAEKER MARC (BE); VANDEKERCKH) 11 June 1998 (1998-06-11)	

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 30-35,49-54

Claims 30-35 and 49-54 relate to compounds characterised by a desirable property, namely that they are modulators or otherwise inhibitors or enhancers of growth cone steering or one of the other listed physiological processes, and to applications of said compounds, without giving a true technical characterization. Moreover, no such compound is defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such speculative claims the wording of which is, in fact, a recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/02373

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9711956 A	03-04-1997	AU 7251496 A	17-04-1997
		CA 2233297 A	03-04-1997
		EP 0854881 A	29-07-1998
		JP 11512611 T	02-11-1999
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